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Comparison of Etest and National Committee for Clinical Laboratory Standards Broth Macrodilution Method for Antifungal Susceptibility Testing: Enhanced Ability To Detect Amphotericin B-Resistant *Candida* Isolates

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The National Committee for Clinical Laboratory Standards (NCCLS) proposed macrobroth reference method (M27P) for susceptibility testing of yeasts is technically difficult. We evaluated Etest, a simple agar-based MIC methodology, as a possible alternative. In studies of six yeast quality control strains, Etest yielded results identical to those obtained by the NCCLS reference method for both amphotericin B and fluconazole. In studies of 91 clinical *Candida* isolates, agreement ± 2 dilutions between the two methods was 95% for fluconazole with phosphate-buffered RPMI 1640 agar and 96 to 97% for amphotericin B with either MOPS (morpholinepropanesulfonic acid)-buffered RPMI 1640 agar or antibiotic medium 3 agar. While the two methods had excellent general agreement, testing of a collection of amphotericin B-resistant isolates demonstrated that, unlike the NCCLS reference method, Etest readily identified the resistant isolates and could do so with a defined medium. Etest is equivalent to the NCCLS proposed method for susceptibility testing of yeasts and superior in its ability to detect amphotericin B resistance.

In vitro susceptibility testing of yeasts is fraught with numerous problems and affected by factors such as media, buffer, and inoculum density (11). A proposed standard (M27P) using RPMI 1640 medium buffered with morpholinepropanesulfonic acid (MOPS) to pH 7.0 in a macrobroth format with incubation for 48 h at 35°C has been published by the National Committee for Clinical Laboratory Standards (NCCLS) (6). This method is time-consuming, expensive, and technically difficult to perform. Modifications of the NCCLS method using a microbroth format will simplify its implementation and have demonstrated results comparable to those for the proposed standard (5). Both of these methods are, however, particularly lacking in their ability to differentiate among amphotericin B-susceptible and -resistant isolates unless antibiotic medium 3 is substituted for RPMI 1640 (9, 11).

Etest, a novel method utilizing a stable gradient of an antimicrobial agent, has been well documented as an accurate and simple method for bacterial susceptibility testing (12), and Etest has recently been shown to produce results comparable to those obtained with the NCCLS method for ketoconazole, itraconazole, and fluconazole (3, 4). We have extended this work by comparing the NCCLS method with Etest for testing of susceptibility of *Candida* isolates to both fluconazole and amphotericin B for a group of clinical isolates as well for a group of recently described quality control strains (7, 8). In addition, having recently demonstrated that the NCCLS reference method has a limited ability to detect amphotericin B-resistant isolates unless antibiotic medium 3 is substituted for RPMI 1640 (9), we also studied the ability of Etest to identify these amphotericin B-resistant isolates.

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MATERIALS AND METHODS

Isolates. Study strains included 91 yeast isolates from blood, a subset of those previously described by Rex et al. (9, 10). A collection of previously described putatively amphotericin B-resistant isolates were used to evaluate the ability of the two test methods to discern resistance to amphotericin B in vitro (2, 9). Six previously described proposed quality control strains were also tested (7, 8).

Antifungal agents. Reference grade lots of amphotericin B (Bristol-Myers Squibb) and fluconazole (Pfizer Pharmaceuticals) were obtained from the manufacturers. By using the procedures specified by the M27P method (6), stock solutions of amphotericin B (in dimethyl sulfoxide) and fluconazole (in water) were prepared and diluted for testing at final concentrations from 0.03 to 16 μg/ml (amphotericin B) and 0.125 to 64 μg/ml (fluconazole) in the macrobroth format. Amphotericin B and fluconazole Etest strips ranging in dilution from 0.002 to 32 μg/ml were kindly provided by AB BIODISK (Solna, Sweden).

Antifungal susceptibility testing. (i) Etest. Five colonies of a *Candida* sp. from a fresh plate were suspended in saline to achieve a turbidity comparable to a 0.5 McFarland standard. This inoculum was swabbed onto the agar plate and allowed to dry for 10 to 15 min before the Etest strips were applied. Media used were RPMI 1640 with L-glutamine (American Biorganics, Niagara Falls, N.Y.) and antibiotic medium 3 (Difco, Detroit, Mich.). Both media were supplemented with an additional 2 g of glucose per 100 ml, and Bacto agar (Difco) was added to produce a final concentration of 1.5 g/100 ml. For testing of amphotericin B, the media were buffered to pH 7.0 with 0.165 M MOPS. Fluconazole was tested only on glucose-supplemented RPMI 1640 buffered to pH 7.0 with 0.1 M phosphate. Plates were incubated at 35°C in plastic bags to retain moisture. MICs were read after 48 h of incubation. For both amphotericin B and fluconazole, Etest MICs could often be read at 24 h, and 24-h MICs did not differ significantly from 48-h MICs (data not shown). However, the MIC was more easily identified after 48 h of growth, especially for slowly growing isolates, and thus the 48-h MICs were used throughout. Amphotericin B MICs were the lowest concentration on the Etest strip at which there was 100% inhibition of the organism. The fluconazole MIC was the least concentration at which there was 80% inhibition of growth as described and illustrated in the Etest technical guide for antifungal susceptibility testing (1).

(ii) Macrobroth. Macrobroth MICs were determined by the NCCLS M27P method (6). As specified by the M27P method, the MIC was read as the con-

TABLE 1. Etest MICs of quality control strains

Organism and antifungal agent	MIC (μg/ml)		
	Recommended	Etest	
		RPMI 1640 agar	AM3 agar[a]
C. parapsilosis ATCC 22019			
Amphotericin B	0.25–1.0	0.25	0.5
Fluconazole	2.0–8.0	4.0	
C. krusei ATCC 6258			
Amphotericin B	0.5–2.0	0.5	2.0
Fluconazole	16–64	32	
C. albicans ATCC 90028			
Amphotericin B	0.5–2.0	0.5	0.5
Fluconazole	0.25–1.0	0.5	
C. albicans ATCC 24433			
Amphotericin B	0.25–1.0	0.25	0.5
Fluconazole	0.25–1.0	0.5	
C. parapsilosis ATCC 90018			
Amphotericin B	0.5–2.0	0.5	0.5
Fluconazole	0.25–1.0	0.5	
C. tropicalis ATCC 750			
Amphotericin B	0.5–2.0	0.5	1.0
Fluconazole	1.0–4.0	2.0	

[a] Fluconazole MICs were not determined on antibiotic medium 3 (AM3).

centration which inhibited growth (amphotericin B) or produced an 80% reduction of turbidity in comparison with the drug-free control (fluconazole).

RESULTS

Etest MICs for NCCLS quality control strains. The MICs obtained by Etest for fluconazole and amphotericin B were compared with the reference MIC ranges for two quality control strains and four reference strains of *Candida* species described by Pfaller et al. (7, 8). The results of testing of these select isolates with the Etest are shown in Table 1 and demonstrate that Etest MICs were found to be within the recommended ranges for all isolates.

Testing of susceptibility to fluconazole. A set of 64 clinical yeast isolates were tested for susceptibility to fluconazole both by NCCLS M27P macrobroth dilution and by Etest on phosphate-buffered RPMI 1640 agar. The overall agreement ± 2 dilutions between M27P and Etest was 95% and ranged from 92% for isolates of *Candida glabrata* to 100% for *C. parapsilosis* (Table 2).

Testing of susceptibility to amphotericin B. A total of 91 isolates were tested for susceptibility to amphotericin B by the NCCLS M27P macrobroth method and Etest. Although RPMI 1640 is a defined medium, it does not support good growth of all yeasts, and growth was more luxuriant on antibiotic medium 3 for the majority of clinical isolates tested. Good agreement was seen between the NCCLS M27P method and Etest with both RPMI 1640 and antibiotic medium 3. Agreement ± 2 dilutions for all isolates was 97 and 96% for RPMI 1640 and antibiotic medium 3, respectively (Table 3).

Identification of amphotericin B-resistant isolates. Results of a comparison of the two methods using several isolates previously tested in an animal model of candidiasis and other clinical isolates with high MICs are shown in Table 4. In particular, the MICs for two isolates that are known to be amphotericin B resistant on the basis of in vivo testing (5W31 and CL2819) were strikingly different from the MIC for the susceptible control from that study (CL524). The MICs for Y533 and Y534, two isolates that appear intermediate in their susceptibility to amphotericin B but that have not been tested in an animal model, were at the upper end of the MIC range for the collection of blood isolates when tested on RPMI 1640 agar and slightly higher than that range when tested on antibiotic medium 3.

DISCUSSION

We have shown that Etest, an accurate and easy-to-perform method for susceptibility testing of bacteria, appears equivalent to the proposed NCCLS reference macrobroth method for testing of *Candida* species susceptibility to antifungal agents. As shown by others (3, 4), the methods are equivalent for the azole antifungal agents, and we have now shown this for amphotericin B as well. Etest MICs for both drugs were in range for the two quality control and four reference isolates of *Candida* species recently identified for use with the NCCLS macrobroth method. In testing a large number of *Candida* isolates, agreement between the two methods was 95 and 96% for fluconazole and amphotericin B, respectively.

Of great interest is the ability of Etest to discriminate between putatively amphotericin B-susceptible and -resistant isolates (Table 4). Compared with the results obtained for the putatively susceptible pool of bloodstream isolates (MIC, 0.125

TABLE 2. Antifungal susceptibilities to fluconazole by M27P and Etest[a]

Species (*n*) [64]	MIC (μg/ml)						% Agreement[d] [95]
	M27P[b]			Etest[c]			
	Range [0.125–>64]	50% [0.5]	90% [32]	Range [0.5–>64]	50% [1]	90% [32]	
C. albicans (23)	0.125–2	0.25	0.5	0.25–4	0.5	1.0	96
C. parapsilosis (12)	0.25–8	1.0	4.0	0.25–4	1	4.0	100
C. tropicalis (14)	0.25–2	0.5	1.0	0.5–4	1	4.0	93
C. glabrata (12)	0.25–>64	16	64	0.5–>64	32	>64	92
C. krusei (2)	32			32–>32			
C. lipolytica (1)	64			128			

[a] Totals are indicated in brackets.
[b] NCCLS M27P macrobroth methodology.
[c] On phosphate-buffered RPMI 1640 agar.
[d] ± 2 dilutions.

TABLE 3. Antifungal susceptibilities to amphotericin B by M27P and Etest[a]

Species (*n*) [90]	M27P[b] MIC (μg/ml)			Etest							
				RPMI 1640				Antibiotic medium 3			
				MIC (μg/ml)			% Agreement[c] [97]	MIC (μg/ml)			% Agreement[c] [96]
	Range [0.125–1.0]	50% [0.5]	90% [1.0]	Range [0.125–1.0]	50% [0.25]	90% [1.0]		Range [0.06–2.0]	50% [0.5]	90% [1.0]	
C. albicans (53)	0.125–1.0	0.5	1.0	0.125–0.5	0.25	0.5	96	0.06–1.0	0.5	1.0	98
C. parapsilosis (10)	0.5–1.0	0.5	1.0	0.125–1.0	0.5	1.0	100	0.06–1.0	0.25	0.5	90
C. tropicalis (13)	0.5–1.0	0.5	1.0	0.125–1.0	0.5	1.0	92	0.06–1.0	0.25	0.5	85
C. glabrata (9)	0.5–1.0	1.0	1.0	0.5–1.0	0.5	1.0	100	0.25–2.0	0.5	1.0	100
C. krusei (2)	0.5–1.0			0.125–0.5				0.25–0.5			
C. lipolytica (1)	1.0			1.0				4			
C. lusitaniae (1)	0.5			0.25				1			

[a] Totals are indicated in brackets.
[b] NCCLS M27P macrobroth methodology.
[c] ± 2 dilutions.

to 1.0 μg/ml; Table 3), the advantage of Etest over the refer ence method is obvious. Further, the ability of Etest to identify resistant isolates was not dependent on the use of the undefined antibiotic medium 3, but was also possible on glucose-supplemented RPMI 1640 agar. Even though modification of M27P by use of antibiotic medium 3 permits identification of the putatively resistant isolates (9), the MIC difference between the susceptible and resistant groups is still only 2 to 3 tube dilutions. On the other hand, Etest generally gave strikingly different MICs for the putatively amphotericin B-resistant isolates.

In conclusion, Etest is comparable to the NCCLS M27P macrobroth method for testing of antifungal susceptibility of clinical yeast isolates to amphotericin B and fluconazole. In addition, Etest appears superior for the detection of resistance to amphotericin B.

TABLE 4. Detection of amphotericin B resistance

Isolate, species	MIC (μg/ml)		
	Macrobroth[a]	Etest	
		RPMI 1640 agar	AM3[b] agar
5W31 (R[c]), *C. lusitaniae*	1–2	4–8	>32
CL2819 (R[c]), *C. lusitaniae*	1–2	32	>32
Y533 (R[d]), *C. lusitaniae*	0.5	1.0	>32
Y534 (R[d]), *C. lusitaniae*	1.0	1.0	4
Y537 (R[d]), *C. albicans*	1	32	>32
MY1012 (R[d]), *C. tropicalis*	2	>32	>32
CL524 (S[c]), *C. parapsilosis*	0.25	0.5	0.5

[a] NCCLS M27P macrobroth methodology.
[b] AM3, antibiotic medium 3.
[c] Known to be susceptible (S) or resistant (R) on the basis of in vivo data (2).
[d] Putative amphotericin B resistance (R) based on in vitro and in vivo data (9).

REFERENCES

1. **AB BIODISK.** 1994. Etest technical guide 4b: antifungal susceptibility testing of yeasts. AB BIODISK, Piscataway, N.J.
2. **Anaissie, E. J., N. C. Karyotakis, R. Hachem, M. C. Dignani, J. H. Rex, and V. Paetznick.** 1994. Correlation between in vitro and in vivo activity of antifungal agents against *Candida* species. J. Infect. Dis. **170:**384–389.
3. **Colombo, A. L., F. Barchiesi, D. A. McGough, and M. G. Rinaldi.** 1995. Comparison of Etest and National Committee for Clinical Laboratory Standards broth macrodilution method for azole antifungal susceptibility testing. J. Clin. Microbiol. **33:**535–540.
4. **Espinel-Ingroff, A.** 1994. Etest for antifungal susceptibility testing of yeasts. Diagn. Microbiol. Infect. Dis. **19:**217–220.
5. **Espinel-Ingroff, A., C. W. Kish, T. M. Kerkering, R. A. Fromtling, K. Bartizal, J. N. Galgiani, K. Villareal, M. A. Pfaller, T. Gerarden, M. G. Rinaldi, and A. Fothergill.** 1992. Collaborative comparison of broth macrodilution and microdilution antifungal susceptibility tests. J. Clin. Microbiol. **30:**3138–3145.
6. **National Committee for Clinical Laboratory Standards.** 1992. Reference method for broth dilution antifungal susceptibility testing of yeasts. Proposed standard M27-P. National Committee for Clinical Laboratory Standards, Villanova, Pa.
7. **Pfaller, M. A., M. Bale, B. Buschelman, M. Lancaster, A. Espinel-Ingroff, J. H. Rex, and M. G. Rinaldi.** 1994. Selection of candidate quality control isolates and tentative quality control ranges for in vitro susceptibility testing of yeast isolates by National Committee for Clinical Laboratory Standards proposed standard methods. J. Clin. Microbiol. **32:**1650–1653.
8. **Pfaller, M. A., M. Bale, B. Buschelman, M. Lancaster, A. Espinel-Ingroff, J. H. Rex, M. G. Rinaldi, C. R. Cooper, and M. R. McGinnis.** 1995. Quality control guidelines for National Committee for Clinical Laboratory Standards recommended broth macrodilution testing of amphotericin B, fluconazole, and flucytosine. J. Clin. Microbiol. **33:**1104–1107.
9. **Rex, J. H., C. R. Cooper, Jr., W. G. Merz, J. N. Galgiani, and E. J. Anaissie.** 1995. Detection of amphotericin B-resistant *Candida* isolates in a broth-based system. Antimicrob. Agents Chemother. **39:**906–909.
10. **Rex, J. H., M. A. Pfaller, A. L. Barry, P. W. Nelson, and C. D. Webb for the NIAID Mycoses Study Group and the Candidemia Study Group.** 1995. Antifungal susceptibility testing of isolates from a randomized, multicenter trial of fluconazole versus amphotericin B as treatment of nonneutropenic patients with candidemia. Antimicrob. Agents Chemother. **39:**40–44.
11. **Rex, J. H., M. A. Pfaller, M. G. Rinaldi, A. Polak, and J. N. Galgiani.** 1993. Antifungal susceptibility testing. Clin. Microbiol. Rev. **6:**367–381.
12. **Sanchez, M. L., and R. N. Jones.** 1992. Etest, an antimicrobial susceptibility testing method with broad clinical and epidemiological application. Antimicrob. Newsl. **8:**1–8.